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Article

Bone Marrow Stem Cell Population in Single and Multiple Level Aspiration

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Abstract: Background: Bone marrow aspiration concentrate (BMAC) has garnered increasing interest due to its potential for healing musculoskeletal injuries. While the iliac crest remains a common harvest site, the aspiration technique's efficacy in offering the highest yield and prevalence of mesenchymal stem cells (MSCs) is controversial. This study aimed to compare two different techniques of bone marrow aspiration over the anterior iliac crest from a single level (SL) versus multiple level (ML). Methods: Anterior iliac crests were selected in 9 adult patients (aged between 31 and 59 years old). Aspiration was achieved using an 11-gauge needle (length: 100 mm; diameter: 2.3 mm) specifically manufactured for bone marrow collection (BD, Becton, US) connected to a 10-mL syringe. On one side, 4cc of bone marrow was aspirated at an SL to a depth of 7cm without changing the needle direction. On the other side, over the same portion of the iliac crest, 1 cc of bone marrow was obtained from ML of different depths during needle retrieval, maintaining a distance of 1cm and changing the tip direction. The samples were blindly sent to the laboratory without indicating whether they came from an SL or ML. Fluorescence-activated cell sorting (FACS) and osteoblast differentiation were analyzed and compared. Results: In the FACS analysis, the SL resulted in a higher population of MSCs that were positive for CD105, CD73, and CD90 and negative for CD34, compared to the ML method. In the process of osteoblast differentiation, it was observed that MSCs exhibited more advanced features of enhanced osteoblastic abilities in the SL method rather than the ML method. Conclusion: An SL aspiration technique at the anterior iliac crest may produce a high-quality bone marrow aspirate. This technique may help obtain specific populations of MSCs with the desired characteristics for use in regenerative therapies for musculoskeletal injuries.

Keywords: bone marrow aspiration concentrate; mesenchymal stem cells; single level; multiple level; musculoskeletal injuries

1. Introduction

The musculoskeletal system plays a vital role in the human body, serving essential functions that involve both structural and metabolic. It supports structure, facilitates movement, protects internal organs, and contributes to metabolic processes related to mineral storage and blood cell production [1,2]. Musculoskeletal injury, the most common cause of disability and daily life limitations, presents a significant socioeconomic challenge [3–5].

Regeneration of musculoskeletal injuries poses challenges in patients with extensive tissue damage, disease, and aging with reduced tissue regeneration capacity. Mesenchymal stem cells

(MSCs) based therapy could be the option for repairing and regenerating musculoskeletal tissues. For example, osteoblasts produce a substantial volume of macromolecules in the bone matrix, including collagenous and noncollagenous proteins that provide a scaffold for matrix mineralization through the deposition of calcium phosphate in the form of hydroxyapatite. Moreover, osteoblasts specialize in matrix production and mineralization for the formation of strong bones. In a mouse model, the origins of osteoblasts include chondrocytes in the growth plate, quiescent bone-lining cells on the bone surface, specialized fibroblasts in the craniofacial structures, and MSCs in the bone marrow. Especially, MSCs in the bone marrow have been shown to be associated with bone development, regeneration, bone anabolism and pathological ossification [6].

Bone marrow aspiration concentrate (BMAC) has become one of orthopedics's most common cell-based musculoskeletal injury therapies. BMAC has the characteristic of a rich source of pluripotent MSCs and growth factors. Moreover, BMAC has anti-inflammatory and regenerative effects for treating musculoskeletal diseases. Many reports have highlighted using BMAC in various orthopedic fields, including osteonecrosis, nonunion of bone fracture, bone defects, and cartilage repair [7–14].

In biological MSCs-based therapy, the primary goal is to improve the methods for obtaining high concentrations of MSCs. Autologous bone marrow obtained from iliac crest bone marrow aspiration has been one of the most utilized sources for cell-based therapies in orthopedics. BMAC contains MSCs, red blood cells, white blood cells, platelets, hematopoietic cells, and nonhematopoietic precursors. The MSC population in BMAC is very few, typically estimated to be 0.01–0.02% of the total cell volume [15,16]. However, the aspiration technique for offering the highest yield is controversial, and the prevalence of MSCs remains obscure. There are various methods for obtaining bone marrow, but consensus still needs to be reached. Because of the aspiration volume can affect the numbers of MSCs, the method of aspiration at a single or multiple level may affect the concentration and quality of MSCs.

In this study, it was hypothesized that if bone marrow were aspirated from at multiple levels, more MSCs could be obtained due to each new aspirated bone marrows compared to bone marrow aspirated in a single level. The purpose of this study was to compare the concentration of MSCs and their potency of BMAC in two different techniques of bone marrow aspiration over the anterior iliac crest from the single level versus the multiple level using Fluorescence-activated Cell Sorting (FACS) analysis and osteogenic differentiation.

2. Materials and Methods

2.1. Antibodies and Reagents

PE-CyTM7 Mouse Anti-Human CD73, APC Anti-Human CD90, FITC Mouse Anti-Human CD105, and PE Mouse Anti-Human CD34 were purchased from BD Biosciences. Fetal bovine serum and Type IV collagenase was purchased from Gibco (Grand Island, NY, USA), ascorbic acid, β -glycerophosphate, and Alizarin Red was purchased from Sigma-Aldrich (St. Louis, MO, USA), and Dulbecco's modified Eagle's medium (DMEM) was purchased from Lonza (Rockland, ME, USA).

2.2. Human Subjects

This study enrolled patients undergoing orthopedic surgery between 30 and 60 years old. The Institutional Review Board of Kyungpook National University Hospital approved using human bone marrow biopsies (IRB File No of KNUH 2021-12-028). We obtained written informed consent from all patients before the surgical procedure. The human sample list is described in Table 1.

Table 1. The human sample list.

Patients	Age	Race	Location of aspiration
1	31	Asian	Iliac crest
2	31	Asian	Iliac crest
3	58	Asian	Iliac crest
4	58	Asian	Iliac crest
5	41	Asian	Iliac crest
6	58	Asian	Iliac crest
7	45	Asian	Iliac crest

2.3. Bone Marrow Aspiration

Bone marrow cells were isolated from the anterior iliac crest using single or multiple level needle puncture aspiration from 9 male patients (mean age 46.7 ±11.8 years, range 31 to 59 years) after receiving written informed consent. Briefly, bone marrow aspirates (4mL) were collected with the syringe with anticoagulant (10 U/mL heparin), and Ficoll-Paque (1.078 g/mL) method was used for cell separation. Anterior iliac crests were selected in 7 adult patients for marrow aspiration sites. Aspiration was achieved with an 11-gauge needle (length, 100 mm; diameter, 2.3 mm) specifically manufactured for bone marrow collection (BD, Becton, USA) connected to a 10-mL syringe (Figure 1).

A



B

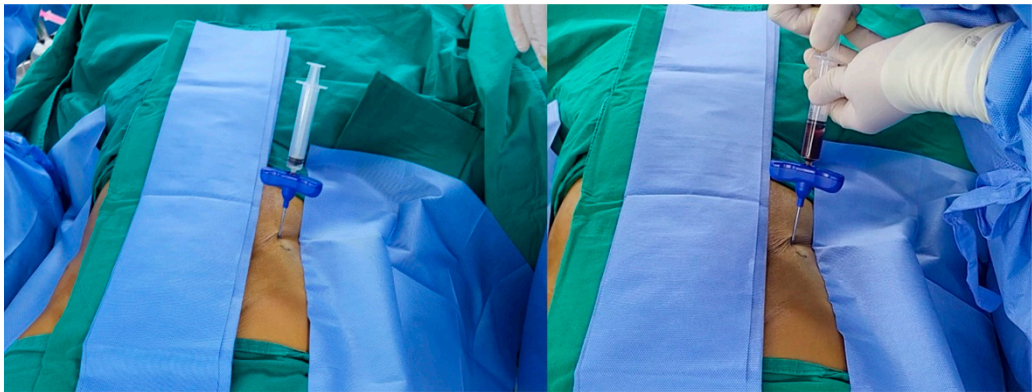
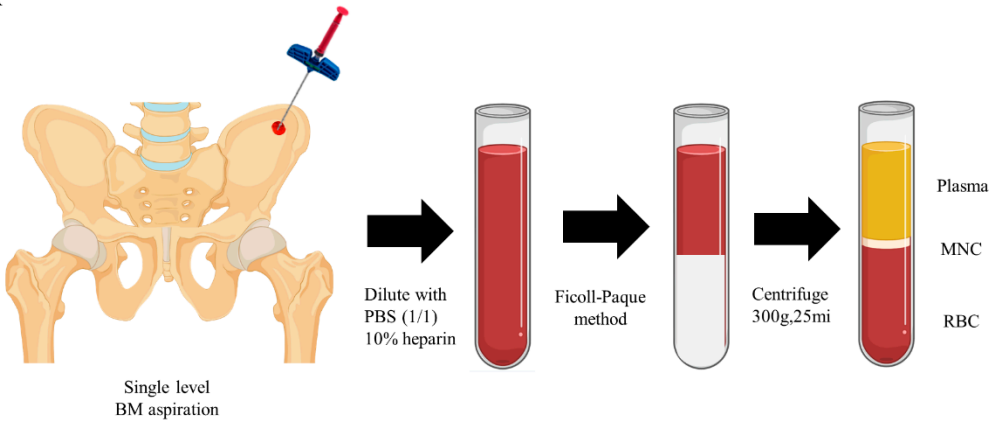


Figure 1. The bone marrow aspiration tool. (A) The bone marrow aspiration tool with an 11-gauge needle specifically manufactured for bone marrow collection & 10-mL syringe. (B) Aspiration was achieved using the aspiration tool. The needle was inserted to the anterior iliac crest and connected to 10-mL syringe.

In single level (SL) samples, 4mL of bone marrow was aspirated at a single level of 7cm depth without changing the needle direction. In multiple level (ML) samples, on the other side over the same portion of the iliac crest, each 1mL of bone marrow was gained at multiple depths while retrieving the needle at a distance of 1cm and changing the tip direction with 180 degrees. A total of 4mL of aspirate was obtained (Figure 2). The samples were blindly sent to the laboratory without the notice of methods.

A



B

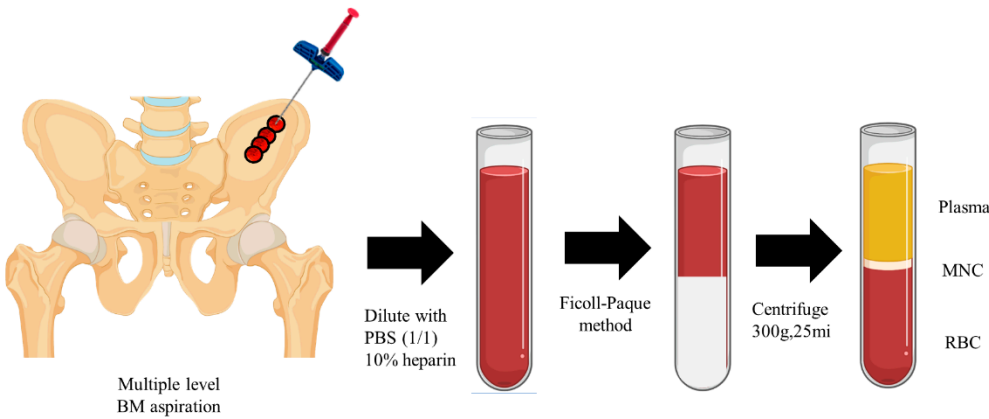


Figure 2. Illustrations of single level (SL) and multiple level (ML) methods for bone marrow aspiration. Bone marrow cells were isolated from the iliac crest through needle puncture aspiration, utilizing single or ML, and obtained bone marrow cells were separated using the Ficoll-Paque method. (A) In SL samples, 4 mL of bone marrow was aspirated at a depth of 7 cm using a single needle insertion without altering its direction. (B) In ML samples, 4 mL of bone marrow was aspirated from multiple depths withdrawn at intervals of 1 cm, changing the tip direction by 180 degrees with each withdrawal obtained for each 1mL bone marrow on the other side of the iliac crest. Abbreviations: BM: bone marrow, PBS: phosphate-buffered saline, MNC: mononuclear cell, RBC: red blood cell.

2.4. Fluorescence-Activated Cell Sorting

We characterized bone marrow cells obtained from single or multiple level isolation methods by their mesenchymal phenotypes. MSC positive and negative markers were used to characterize the MSC phenotyping. For Fluorescence-activated Cell Sorting (FACS), the bone marrow cells were washed with FACS buffer containing 2.5% BSA and 0.25% sodium azide (Sigma A) in PBS and incubated with positive markers of CD73 (PE-CyTM7-conjugated), CD90 (APC-conjugated), CD105 (FITC-conjugated), and negative markers of CD34 (PE-conjugated) for 1 hour at 4 °C then washed briefly in FACS buffer. FACS data were obtained using a FACS Calibur II (BD Biosciences).

2.5. Cell Seeding and Osteogenic Differentiation

For osteogenic differentiation, bone marrow-derived mononuclear cells (MNCs) were seeded in the 24-well plate at a density of $1 \times 10^4/\text{cm}^2$ and incubated in an osteogenic medium containing alpha-minimum essential medium (α -MEM, Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS, Gibco BR, Gaithersburg, MD L), ascorbic acid (50 $\mu\text{g}/\text{mL}$) and beta-glycerophosphate (10 mM) for osteoblast differentiation [17]. MSCs was cultured in an osteogenic medium for 28 days with medium change three times a week.

2.6. Alizarin Red Staining

Osteoblast mineralization was detected by Alizarin Red staining. After 28 days, cells were washed three times with PBS, fixed with 4% PFA (paraformaldehyde) for 10 min at room temperature, and stained with 2% alizarin red (pH 4.2) to evaluate calcium deposition in differentiated osteoblast. Samples were washed in filtered deionized water and analyzed with a Leica microscope.

2.7. Statistical Analysis

Statistical analyses were performed by GraphPad Prism 9 (GraphPad, San Diego), and data were expressed as mean $\pm$ standard deviation. Statistical significance was analyzed using a student's t-test and indicated as follows: * $p < 0.05$, *** $p < 0.01$.

3. Results

3.1. Advanced MSC Population in a Single Level Bone Marrow Aspiration

Separate MSCs were analyzed using FACS assay to assess the MSC population in single and multiple level using bone marrow aspiration methods. For quantification, high purity of MSC population CD73 (PE-CyTM7-conjugated), CD90 (APC-conjugated), and CD105 (FITC-conjugated) as a positive marker, while CD34 (PE-conjugated) was used as negative markers in FACS analysis (Figure 3A). First, we checked the CD73+ cell numbers from the single and multiple level of bone marrow aspiration using FACS analysis. FACS analysis of the bone marrow cells obtained from each group revealed that multiple level bone marrow aspiration significantly decreased CD73 to 81.3% compared to single level bone marrow aspiration. To exclude the hematopoietic stem cells and adipose tissue derived-MSCs, we determined the cells CD73+ while CD34- using FACS analysis. Compared with single level bone marrow aspiration, multiple level bone marrow aspiration significantly decreased CD73+ and CD34- to 82.6%. In addition, to determine the high purify bone

marrow derived-MSCs quantification, we determined the cells both CD90+ and CD105+ among the CD73+ while CD34- gated cells using FACS analysis. Compared with single level bone marrow aspiration, multiple level bone marrow aspiration significantly decreased CD90+ while CD73+ and CD34- to 64.3%; however, there was no significance in the CD105+ while CD73+ and CD34- cells. Finally, the number of CD90+ and CD105+ while CD73+ and CD34- cells in multiple level bone marrow aspiration was significantly decreased to 57.8% compared with single level bone marrow aspiration (Figure 3B, Table 2).

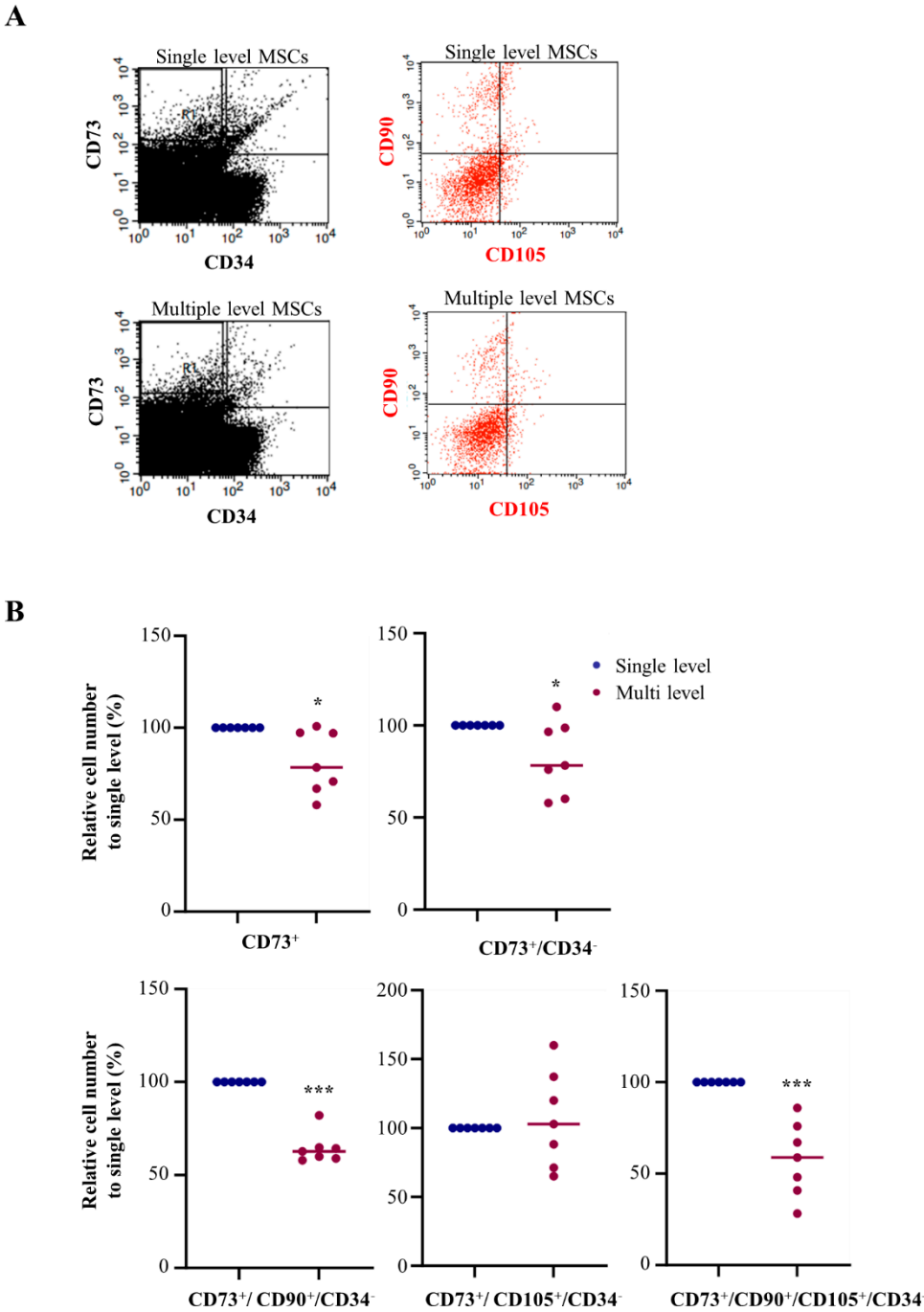


Figure 3. MSC population of samples from a single level and multiple level. Fluorescence-activated cell sorting (FACS) analysis of the expression of surface antigens on MNCs isolated from SL or ML bone marrow aspiration. A minimum amount of 1 X 10⁶ cells was examined for the FACS analysis. (A) The CD73+ and CD34- expression MNCs were measured and gated the cells for specific co-expression of CD73+ and CD34- cells (black panel). The gated cells were examined for co-expression for surface antigens CD90 and CD105 and counted as pure MSCs population (Red channel). (B) The CD73+, CD73+/CD34-, CD73+/CD90+/CD34- and CD73+/CD90+/CD105+/CD34- cells were counted using FACS analysis. Abbreviation, MSC: mesenchymal stem cell, *p <0.05, ***p <0.01.

Table 2. Relative MSCs numbers of multiple level compared with single level.

Markers	Relative MSC number to single site (%)	p-value
CD73 ⁺	81.3 ± 17.04	<0.05
CD73 ⁺ /CD34 ⁻	82.6 ± 19.92	<0.05
CD73 ⁺ /CD90 ⁺ /CD34 ⁻	64.3 ± 8.25	<0.001
CD73 ⁺ /CD105 ⁺ /CD34 ⁻	106.4 ± 34.86	n.s.
CD73 ⁺ / CD90 ⁺ /CD105 ⁺ /CD34 ⁻	57.8 ± 20.29	<0.001

3.2. Advanced MSC Population in a Single Level Bone Marrow Aspiration

Next, we performed in vitro osteogenic differentiation using MNCs derived single and multiple level aspiration. Alizarin Red analysis of the bone marrow cells obtained from each group revealed that multiple level bone marrow aspiration significantly decreased bone mineralization to 47% compared to single level bone marrow aspiration. Mesenchymal stem cells of single level bone marrow aspiration were more rapidly differentiated into osteoblasts and promoted mineralization effects than those of multiple level bone marrow aspiration in Alizarin Red staining (Figure 4A). The quantification of osteoblast mineralization areas was also showed significantly enhanced in SL compared with ML (Figure 4B, Table 3). These results indicate that single level bone marrow aspiration has promoted ability in osteogenic differentiation.

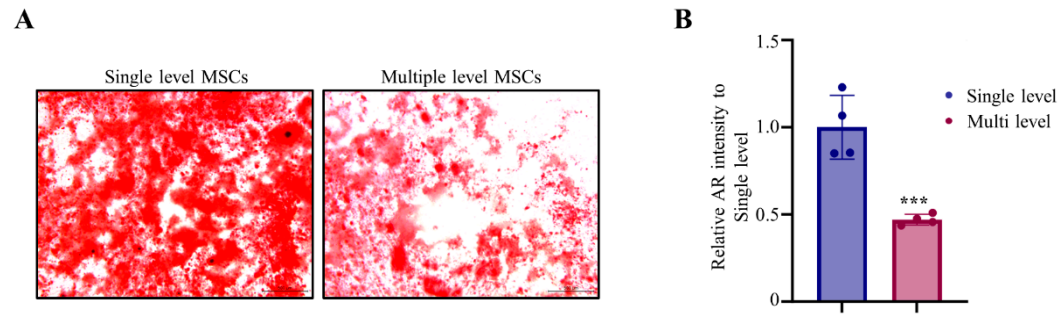


Figure 4. Advanced osteoblast mineralization in SL MSCs. Harvested MNCs derived from SL and ML were used for osteoblast differentiation. Osteoblast mineralization was confirmed by the (A) Alizarin red staining and (B) quantified by the Bioquant Osteo 2019 v19.9.60 program at day 28 after osteogenic differentiation. Abbreviations: AR: Alizarin Red, MSCs: mesenchymal stem cells, SL: single-level, ML: multiple-levels, *p < 0.05, ***p < 0.01.

Table 3. Relative Alizarin Red intensity of samples from ML compared with SL.

	Relative AR intensity to a single site (%)	p-value
Compared with multiple sites	47 ± 17.04	<0.05

AR: Alizarin Red.

4. Discussion

This study found that bone marrow aspiration from a single level showed a higher population of MSCs than those of multiple level one. In osteoblast differentiation, MSCs exhibited more advanced features of osteoblastic abilities in the single level rather than the multiple level method. These results indicate that the single level bone marrow aspiration showed higher yield and

prevalence of MSCs and advanced osteoblastic differentiation than the multiple level technique. The changes in depth during aspiration could affect the concentration and quality of MSCs and it is thought that the concentration of MSCs varies depending on the depth and is lower when performing aspiration BM at a shallower depth.

In orthopedic surgery, MSCs could be the options for regenerative treatment in various problems, and several studies are ongoing. MSCs enable the regeneration of musculoskeletal tissues, especially bone marrow stromal cells (BMSCs), which can differentiate into several lineages, including chondrocytes, osteoblasts, and adipocytes. BMSCs were commonly used sources of MSCs because of the easy collection method. Although BMSCs can be used as a cell suspension after being expanded by culture or as a concentrate, the clinical practice may be limited, considering the problems related to cell manipulation and expansion. However, BMAC can overcome the need for culture expansion, and minimal manipulation can be applied in a one-step treatment. It has been widely utilized in the clinical practice of orthopedic areas. It also could prevent the risk of allogenic disease and infection. Therefore, the clinical use of BMAC will expand further.

MSCs from BMAC could be applied in various areas of orthopedics. First, it could be used in the treatment of osteonecrosis. Hernigou et al [16] suggested that ONFH may be the disease of bone cells and/or MSCs. The numbers and activity of MSCs decreased in bone marrow in patients with ONFH. Moreover, the number of progenitor cells in patients who underwent corticosteroid treatment was lower than in patients with a different underlying etiology. In this situation, BMAC could be the treatment option. Autologous MSCs exhibit anti-inflammatory, immunomodulatory, and tissue-protective "trophic" effects [18]. BMAC also applies to osteoarthritis (OA) patients, especially in the knee. The treatment options were various in patients with knee OA. From non-pharmacological treatment, including weight loss and physical treatment, to pharmacological treatment, including oral medication and intraarticular injection, the various options can be applied by non-surgical treatment. Recently, the treatment using MSCs has been considered the new treatment option for OA due to its structural contributions to tissue repair, immunomodulatory and anti-inflammatory actions through direct cell-to-cell interaction, or secretion of bioactive factors. Finally, BMAC could be used in the treatment of fractures and nonunion. In the musculoskeletal system, bone is a dynamic self-healing tissue that continuously remodels in response to mechanical loadings throughout life. However, bone remodeling cannot repair between 5 and 10% of all bone fractures, resulting in delayed or failed healing in extensive traumatic injuries [19,20]. In clinical practice, the preferred approach for treating fractures is autologous iliac crest bone graft replacement, considered the gold standard for treatment [21]. However, the application limitation was in the significant bone defect, and the donor site morbidity could be a problem after autologous bone graft. The other methods for nonunion, including allograft, bone substitutes, demineralized bone matrix (DBM), and ultrasound and pulsed electromagnetic field, could be used for bone healing. Osteogenic stem cells in bone marrow have the biological efficacy of cancellous bone [22,23]. Therefore, they would be applied to the treatment of fracture or nonunion [24,25].

In biological MSCs-based therapy, the primary goal is to improve the methods for obtaining high concentrations of MSCs. Autologous bone marrow obtained from iliac crest bone marrow aspiration has emerged as one of the most utilized sources for cell-based therapies in orthopedics. BMAC contains MSCs, red blood cells, white blood cells, platelets, hematopoietic cells, and nonhematopoietic precursors. The MSC population in BMAC is rare and typically estimated to be 0.01-0.02% of the total cell volume.^{15,16} The osteogenic capacity correlated with the cell concentration [26], and the efficacy could be related to the number of progenitors.¹⁶ Therefore, efforts to increase the progenitor cells and maximize the MSC content at the time of the aspiration are essential, and accurate methods for bone marrow aspiration are very important for promising therapeutic options for orthopedic treatments. There still needs to be a consensus on the BMAC aspiration method. Numerous research studies have concentrated on enhancing bone regeneration by applying MSCs based therapy. Oliver et al [27] reported no differences in cell concentration between the single site and multiple sites harvesting systems, but the pain was decreased in the single site technique. In this study, the single level bone marrow aspiration technique obtained a higher population of MSCs with

MSC positive markers of CD73, CD90, and CD105, while negative MSC markers of CD34 compared with multiple level bone marrow aspiration technique. This is thought that changes in position, depth and direction during aspiration affect the concentration and quality of MSCs. Obtaining a high-quality bone marrow aspirate for clinical application could be helpful.

The standardized measurement and reporting on the yield and quality of bone marrow aspirate samples, the colony-forming unit, and the combination of different markers have been suggested to characterize MSCs present within BMAC. The International Society for Cellular Therapy (ISCT) has established standardized terminology and minimal criteria for characterized MSCs features involved, has plastic-adherent ability in standard culture system in vitro, expressed surface markers CD73, CD90, and CD105 and lack expression of hematopoietic markers CD34, CD45, CD14, CD19, and HLA-DR (human leukocyte antigen-antigen D-related), and can differentiate to osteoblasts, adipocytes, and chondroblasts in vitro culture system [28–31]. Our data determined that single level bone marrow aspiration technique obtained a higher population of MSCs with MSC positive markers of CD73, CD90, and CD105 while negative MSCs markers of CD34 compared with multiple level bone marrow aspiration technique.

The limitation of this study was a small number of patients. The volume of BM aspirate, the size of the syringe, and the pressure during aspiration could change the results. However, this study evaluated only the aspiration method between single and multiple level. Further larger-scale studies considering various methods of aspiration should be evaluated.

5. Conclusions

A high-quality bone marrow aspirate could be obtained through a single level aspiration technique at the anterior iliac crest rather than a multiple level aspiration technique. This approach is known for its effectiveness in obtaining specific populations of mesenchymal stem cells with the desired characteristics, making them valuable for regenerative therapies for musculoskeletal injuries.

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Institutional Review Board Statement: The study received approval from the Institutional Review Board (IRB) of Kyungpook National University Hospital under protocol number KNUH 2021-12-028. All methodologies adhered to pertinent guidelines and regulations. All patients signed informed consent before participating in this study.

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Data Availability Statement: Data is provided within the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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